AFOSR Grant Number 81-0087
Annual Technical Report
December 1982

DEVELOPMENT AND USE OF ANUCLEATE BACTERIAL CELLS TO ASSAY THE IN VITRO ACTIVITY OF POLLUTANTS

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Controlling Office: Air Force Office of Scientific Research/NL
Bolling Air Force Base, DC 20332

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

12

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER **AFOSR-TR- 83-0442**	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) DEVELOPMENT AND USE OF ANUCLEATE BACTERIAL CELLS TO ASSAY THE IN VITRO ACTIVITY OF POLLUTANTS		5. TYPE OF REPORT & PERIOD COVERED Interim Report for period April 1, 1981-31 July 1982
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) John N. Reeve		8. CONTRACT OR GRANT NUMBER(s) AFOSR 81-0087
9. PERFORMING ORGANIZATION NAME AND ADDRESS Ohio State University Department of Microbiology 484 W. 12th Avenue; Cols., OH 43210		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102F 2312/A5
11. CONTROLLING OFFICE NAME AND ADDRESS United States Air Force Air Force Office of Scientific Research /NL Building 410, Bolling AFB, D.C. 20332		12. REPORT DATE December 7, 1982
		13. NUMBER OF PAGES 33
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) unclassified
		15a. DECLASSIFICATION DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

DTIC ELECTE S MAY 23 1983 D E

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

T7 0.3 gene
misincorporation in vivo
mistranslation in vivo
assay for mistranslation

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The T7 0.3 gene product (0.3 protein) was purified by a modification of the published procedure (2), and used to raise antibody to this protein. A radioimmune precipitation (RIP) assay was developed which could be used to estimate the increased misincorporation of cysteine into 0.3 protein. Parameters of the RIP assay were varied to make the RIP-polyacrylamide gel electrophoresis (RIP-PAGE) assay specific for the 0.3 protein. A single protein band was, however, never achieved although increased misincorporation of cysteine into the 0.3 protein can now be estimated by RIP-PAGE combined with scanning densitometry.

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AD A123378

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PROGRESS REPORT

Grant #AFOSR 81-0087
Title: Development and use of anucleate bacterial cells to assay the *in vivo* activity of pollutants

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Report for research undertaken between April 1, 1981 - August 1, 1982

I. Research undertaken between April 1, 1981 - February 1, 1982
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I. Report for research undertaken between April 1, 1981 - February 1, 1982

A. Abstract

Experiments were undertaken to determine if misincorporation of ^{35}S-cysteine into non-cysteine containing T7 proteins could be quantitated by autoradiography. It was found that a minimum of 10^7 radioactive sulphur atoms were required to sufficiently expose X-ray film so that scanning densitometry could be used for quantitation. Calculations based on this determination indicated that the expected misincorporation of ^{35}S-cysteine into T7 proteins in infected minicells would be insufficient to permit autoradiography to be used as the assay for misincorporation. It was therefore decided to develop a more sensitive assay based on immunoprecipitation of specific T7 proteins synthesized in infected minicells. A modification of a published procedure to isolate T7 0·3 gene product has been developed and we are now in the position to raise antibody to this purified protein.

Experiments have begun to determine if it is feasible to construct a recombinant bacteriophage containing a large (1000-3000 base-pairs) stretch of homopolymer i.e. poly dA-dT or poly dG-dC. The intent is to develop a probe for detection of mistranscription in vivo. These experiments have so far been unsuccessful. It appears that although monopolymers can be constructed in vitro they are not stable in vivo.

B. Detection of ^{35}S-cysteine incorporation into T7 polypeptides

Anucleate bacterial cells (minicells) were infected with T7 and allowed to incorporate ^{35}S-methionine or ^{35}S-cysteine. The radioactively labeled polypeptides, synthesized in infected minicells, were separated by electrophoresis and the resulting gels used to expose X-ray film using the technique of fluorography. As previously observed three T7 proteins (products of genes 0·3, 9 and 16) were not labeled by incorporation of ^{35}S-cysteine, i.e. they do not contain cysteine residues. Sample volumes were increased to increase the amounts of radioactive proteins on the gels. We were still unable to detect ^{35}S-cysteine incorporation into 0·3, 9 or 16 proteins at sample sizes so large that gel resolution was impaired. Faint exposures of the X-ray film were obtained using ^{35}S-cysteine incorporation into 0·3 protein by exposing the gel to the film for 8-12 weeks. This is not, however, a practical laboratory procedure.

Figure 1 shows the results of exposing known amounts of ^{35}S-cysteine to X-ray film. It can be calculated that a minimum of 10^7 ^{35}S-atoms are required to produce an exposure suitable for quantitation by scanning densitometry in two days. Assuming a random amino-acid misincorporation rate of 1 per 10,000 peptide bonds (1) and a polypeptide length of 116 amino-acids (0.3 protein (2)) then one

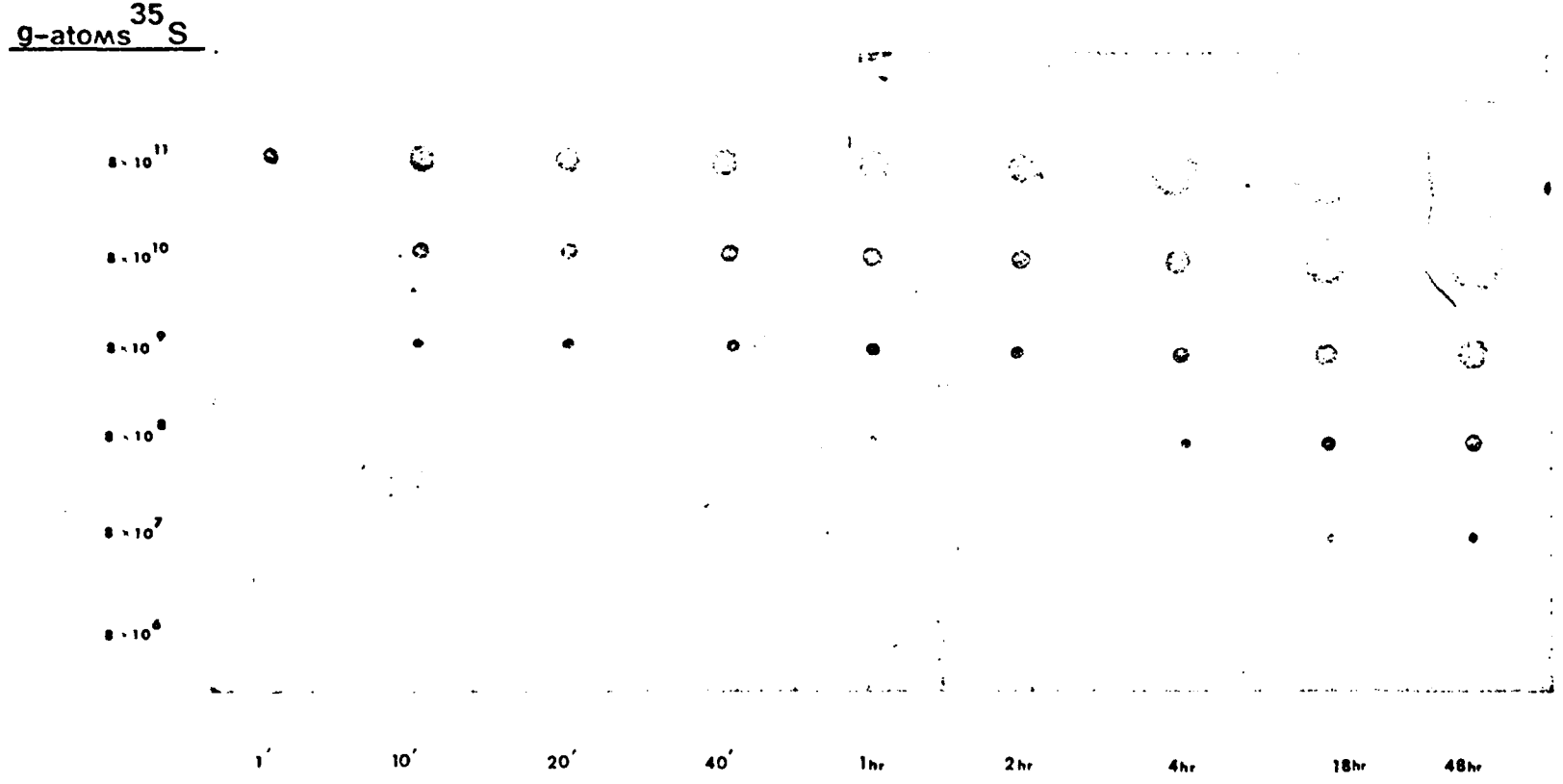


Figure 1. Known amounts of ^{35}S-cysteine were impregnated into a dryed polyacrylamide gel prepared for fluorography. The gel was exposed to X-ray film for time periods indicated below each series of spots. The number of atoms of ^{35}S in each sample are given at the side of the figure.

^{35}S-cysteine should be misincorporated into approximately one 0·3 molecule per 2000 molecules synthesized. (This assumes that misincorporation of cysteine occurs at 1/20th the rate of total amino-acid misincorporation.)

If one 0·3 molecule is labeled per 2000 molecules synthesized and 10^7 ^{35}S-atoms are required for detection then a minimum of $2x10^{10}$ 0·3 molecules must be loaded onto the gel for there to be a reasonable probability that the misincorporation of ^{35}S-cysteine will be detected by our autoradiography procedures. Our routine procedures employ $1x10^9$ minicells per sample and therefore each T7 infected minicell would have to synthesize 20 molecules of 0·3 protein to obtain the $2x10^{10}$ molecules per sample needed for minimum detection of ^{35}S-cysteine misincorporation. Synthesis of 20 molecules of 0·3 protein per infected minicell should occur; however, our experimental results indicate that we do not routinely observe ^{35}S-cysteine labeling of 0·3 protein. We must, therefore, assume that one or more of the quantitative assumptions used above in making calculations is incorrect. Attempts to stimulate ^{35}S-cysteine incorporation by addition of streptomycin (known to increase mistranslation by a factor of 2 to 3) to give detectable incorporation were not successful. The conclusion of these studies was that quantitation of ^{35}S-cysteine incorporation by direct autoradiography of extracts of T7 infected minicells is not sufficiently sensitive to develop as a routine procedure.

C. Purification of 0·3 protein

Based on our calculations that quantitation of ^{35}S-cysteine misincorporation into 0·3 protein could not be successfully obtained by scanning densitometry we decided to purify the 0·3 protein so that antibody could be raised against the protein and used to specifically precipitate 0·3 protein from T7 infected minicells. The misincorporation of ^{35}S-cysteine could then be quantitated by scintillation counting. In addition, antibody against 0·3 protein could be used to precipitate 0·3 protein synthesized in T7 infected cells, as well as in minicells, increasing the potential alternative systems in which 0·3 protein could be used to assay errors in protein synthesis.

A procedure for purification of 0·3 protein has been published (2) and we initially followed the procedure precisely as described. In essence the purification depends on the ability of 0·3 protein to bind to DEAE even in the presence of 0·3M NH_4Cl plus the unusual property that 0·3 protein is soluble in ethanol. Our experiments confirmed that 0·3 can be separated from most proteins in an extract of T7 infected cells by binding to DEAE at 0·3M NH_4Cl and elution at 0·7M NH_4Cl. Our problems arose in our attempts to repeat the published procedures to dissolve 0·3 protein in ethanol following TCA precipitation. A very time consuming series of experiments were undertaken varying salt concentration, pH, sequence of additions, etc. to obtain pure 0·3 protein as determined by SDS-gel electrophoresis using silver nitrate staining. We now have resolved these problems and have 0·3 protein in sufficient amounts to begin vaccination procedures for antibody production. Figure 2 is a flow chart of our procedure for isolating 0·3 protein from T7 infected cells.

Figure 2. Purification of T7 0·3 protein. Modification of the procedure described by Mark and Studier (1981).

Extract of T7 H3; lam193;LG3 infected E. coli cells
↓
DNase digestion. Bring to 0·3M NH_4Cl
↓
Slow speed centrifugation
High speed centrifugation
↓
Pass supernatant through DEAE column in 0·3M NH_4Cl. Most proteins do not bind.
↓
Elute with 0·3M → 1M NH_4Cl gradient
↓
Test fractions for 0·3 protein by running 10-20% polyacrylamide gel and $AgNO_3$ staining
↓
Add 10% TCA to 0·3 containing fractions
Collect precipitate
Dissolve in Tris buffer + 0·3M NH_4Cl
↓
Add 4 volumes ethanol; pH2
Discard precipitate
↓
Add 8 volumes ethanol
↓
Discard supernatant
Dissolve pellet (pure 0·3) in 0·1M Tris buffer

D. Construction of a lambda based recombinant

Only a limited number of experiments have been undertaken on this part of the project as Dr. Alice Desmyter, who was hired to carry out these experiments, did not arrive in Columbus until December 1981. The problem of recruiting trained personnel for recombinant DNA studies was the basis for the no-cost extension granted to the project. Our experiments to date have been designed to determine the procedural conditions to synthesize long (1000-3000) base-pair homoduplexes using DNA polymerase I in the absence of template DNA and using terminal transferase. Based on the results of incorporation of radiochemically labeled precursors and gel electrophoresis of products it does appear that molecules of the appropriate length can be synthesized in vitro. We have not, so far, been able to clone these molecules into lambda vectors. The preliminary experiments already completed indicate that homopolymers may not be stable in vivo. A similar conclusion has been reached by Prof. R.D. Wells (University of Wisconsin; Personal communication 1981).

Report for research undertaken between Feb. 1, 1982 - Aug. 1, 1982

A. Abstract

The T7 0.3 gene product (0.3 protein) was purified by a modification of the published procedure (2), and used to raise antibody to this protein. A radioimmune precipitation (RIP) assay was developed which could be used to estimate the increased misincorporation of cysteine into 0.3 protein. Parameters of the RIP assay were varied to make the RIP-polyacrylamide gel electrophoresis (RIP-PAGE) assay specific for the 0.3 protein. A single protein band was, however, never achieved although increased misincorporation of cysteine into the 0.3 protein can now be estimated by RIP-PAGE combined with scanning densitometry.

Experiments to determine the normal level of mistranslation in vivo are currently in progress. All necessary preliminary experiments for these assays have been completed. We have devised a labeling medium, determined the minimum saturating levels of cysteine and developed satisfactory methods to purify small amounts of labeled 0.3 protein, to demonstrate nonconversion of cysteine to methionine and to reliably determine the specific activity of purified, labeled 0.3 protein.

B. Detection of ^{35}S-cysteine incorporation into 0.3 protein by radioimmune precipitation

1. Preliminary experiments. Rabbit antibody was raised to purified 0.3 protein. This was allowed to react with ^{35}S-methionine labeled lysates of T7-infected E. coli cells. In our initial RIP experiments protein A-containing S. aureus was used to precipitate immune complexes. On visualization of the immune complexes by fluorography following PAGE, 0.3 protein was the major band present; however, other polypeptides were also present. Exhaustive attempts were made to remove these extraneous polypeptides by varying conditions of the assay. Improvements were seen, especially when protein A Sepharose was used instead of S. aureus for precipitation of immune complexes (fig. 3). We were, however, unable to completely eliminate the other polypeptides. This may be due to nonspecific adherence of these polypeptides to protein A Sepharose or to the presence in our 0.3 antiserum of rabbit antibody to E. coli or to other T7 proteins.

2. Specificity of the RIP-PAGE assay for the 0.3 protein. Specificity was demonstrated by reacting normal or immune serum with lysates of E. coli strains infected with a T7 amber mutant (CR10b) in the 0.3 gene. These strains were either capable or incapable of suppressing the nonsense mutation in the 0.3 gene (fig. 4). With normal serum, protein was not seen in the 0.3 protein position in either case, but with immune serum the 0.3 protein was seen, and only when the nonsense mutation-suppressing strain was used.

3. Increase in misincorporation of cysteine into 0.3 protein with drugs. The RIP-PAGE assay was used to detect an increase in ^{35}S-cysteine incorporation into the 0.3 protein after treatment with streptomycin or gentamicin. An increase in incorporation was detected by both scintillation counting and autoradiography using either drug at all concentrations tested (5, 10, 20 and 40 ug/ml). In the absence of drugs ^{35}S-cysteine incorporation into 0.3 protein was detectable by autoradiography in only some experiments and in those cases exposure times in excess of 2 weeks were required. When

RIP-PAGE: ^{35}S-Methionine Labelled E. coli B Extract

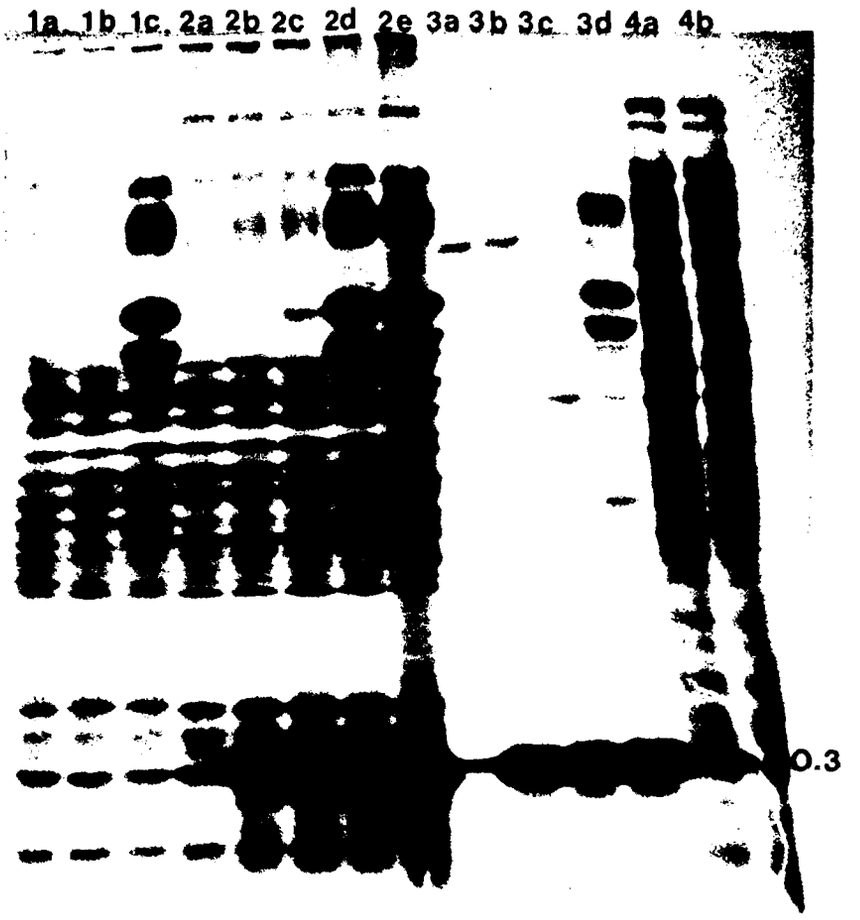
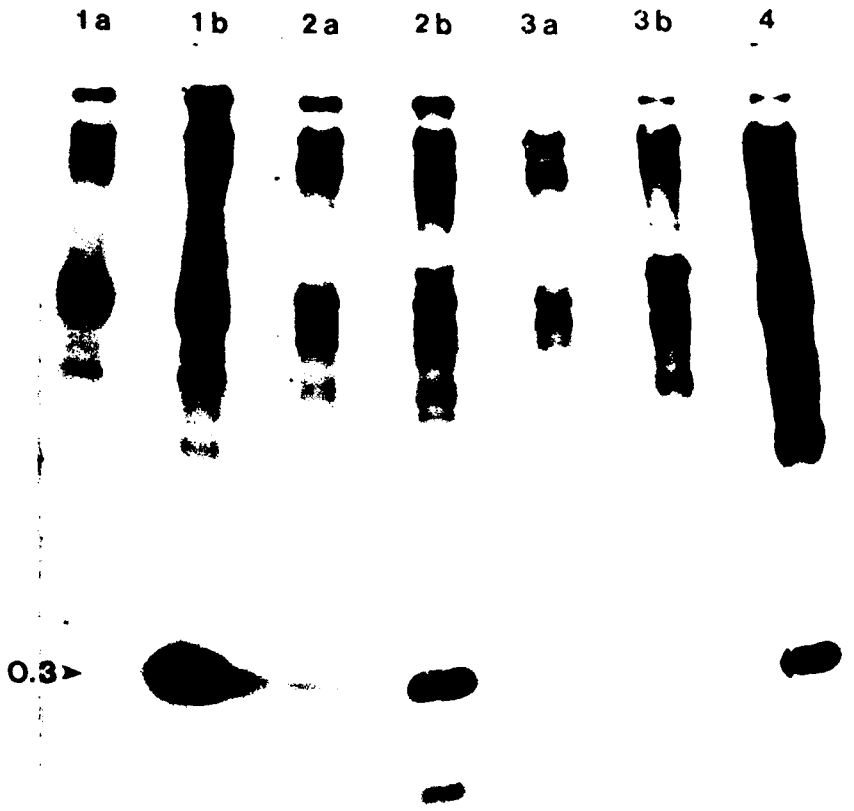


FIGURE 3

1a = Uninfected E. coli + normal serum + S. aureus
1b = Uninfected E. coli + immune serum 1 + S. aureus
1c = Uninfected E. coli + immune serum 2 + S. aureus
2a = T7 infected E. coli + normal serum + S. aureus
2b = T7 infected E. coli + absorbed immune serum 1 + S. aureus
2c = T7 infected E. coli + immune serum 1 + S. aureus
2d = T7 infected E. coli + absorbed immune serum 2 + S. aureus
2e = T7 infected E. coli + immune serum 2 + S. aureus

3a = T7 infected E. coli + normal serum + protein A Sepharose
3b = T7 infected E. coli + absorbed immune serum 1 + protein A Sepharose
3c = T7 infected E. coli + immune serum 1 + protein A Sepharose
3d = T7 infected E. coli + immune serum 2 + protein A Sepharose
4a = Extract of T7 infected E. coli B cells used in this experiment
4b = Extract of T7 infected E. coli B cells used in this experiment

FIGURE 4



T7 infected E. coli labelled with ^{35}S-methionine was allowed to react with normal or immune serum and then protein A Sepharose.

1a = T7 infected E. coli B + normal serum
1b = T7 infected E. coli B + immune serum
2a = T7 (0.3 amber mutant) infected Su^+ strain of E. coli + normal serum
2b = T7 (0.3 amber mutant) infected Su^+ strain of E. coli + immune serum
3a = T7 (0.3 amber mutant) infected Su^0 strain of E. coli + normal serum
3b = T7 (0.3 amber mutant) infected Su^0 strain of E. coli + immune serum
4 = Extract of T7 infected E. coli

streptomycin or gentamycin were added, however, ^{35}S-cysteine incorporation was always detectable and the darkness of the band (amount of ^{35}S-cysteine incorporation) was proportional to the concentration of the drug used (fig. 5). To quantitate the increase in misincorporation of ^{35}S-cysteine into 0.3 protein we will subject this autoradiogram to scanning densitometry. An isolated cysteine-containing protein will be used to correct for the overall decrease in protein synthesis resulting from the use of gentamicin at these high concentrations.

C. Determination of the normal mistranslation level in vivo

To estimate the increased error frequency in translation in vivo when drugs or toxic chemicals are present, it is necessary to know the natural error level. The T7 0.3 protein system provides an advantage over other systems used to estimate mistranslation levels in vivo. The T7 0.3 protein is made only after T7 infection and therefore all the 0.3 protein made is under the conditions of the experiment, i.e., all 0.3 protein synthesized is labeled. It is therefore unnecessary to determine the amount synthesized during the labeling period relative to the amount existing before label was added. In addition, we have simplified the purification procedure for 0.3 protein and made it suitable for the small amounts of protein obtained in labeling experiments.

1. Purification of small amounts of labeled 0.3 protein. Two methods were compared: affinity chromatography using rabbit anti- 0.3 antibody conjugated to CNBr-activated Sepharose 6B and DEAE cellulose anion exchange chromatography followed by ethanol extraction (scaled down from our procedure to purify milligram amounts of 0.3 protein). We found that the 0.3 protein, eluted from the immunoaffinity column, was impure and required additional ethanol extraction steps for a complete purification. In addition, the capacity of the immunoaffinity columns was much less than that of the DEAE columns and not sufficient to retain all the 0.3 protein made in a labelling experiment. We, therefore, decided against the use of immunoaffinity chromatography and have used anion exchange chromatography for the initial purification steps.

2. Experimental conditions necessary for calculations of mistranslation levels. To calculate the normal mistranslation frequency, i.e., the number of cysteine residues per molecule of 0.3 protein, it is necessary to know the specific activity of cysteine available to the cell for protein synthesis. This means that the amount of radioactively labelled cysteine provided should not be diluted by endogenous cysteine synthesis nor should the precursor ^{35}S-cysteine be converted to methionine. An excess of methionine must be provided to block cysteine conversion into methionine by feedback inhibition and the endogenous pathway of cysteine biosynthesis must be repressed by providing a saturating level of cysteine in the absence of sulfate. Experiments were performed to determine minimum saturating levels of cysteine and methionine. In Figures 6 and 7 cysteine and methionine uptake can be seen to be at the saturating level at 5×10^{-4} M. Additional experiments showed that the minimum saturating level for both amino acids was 2×10^{-4} M.

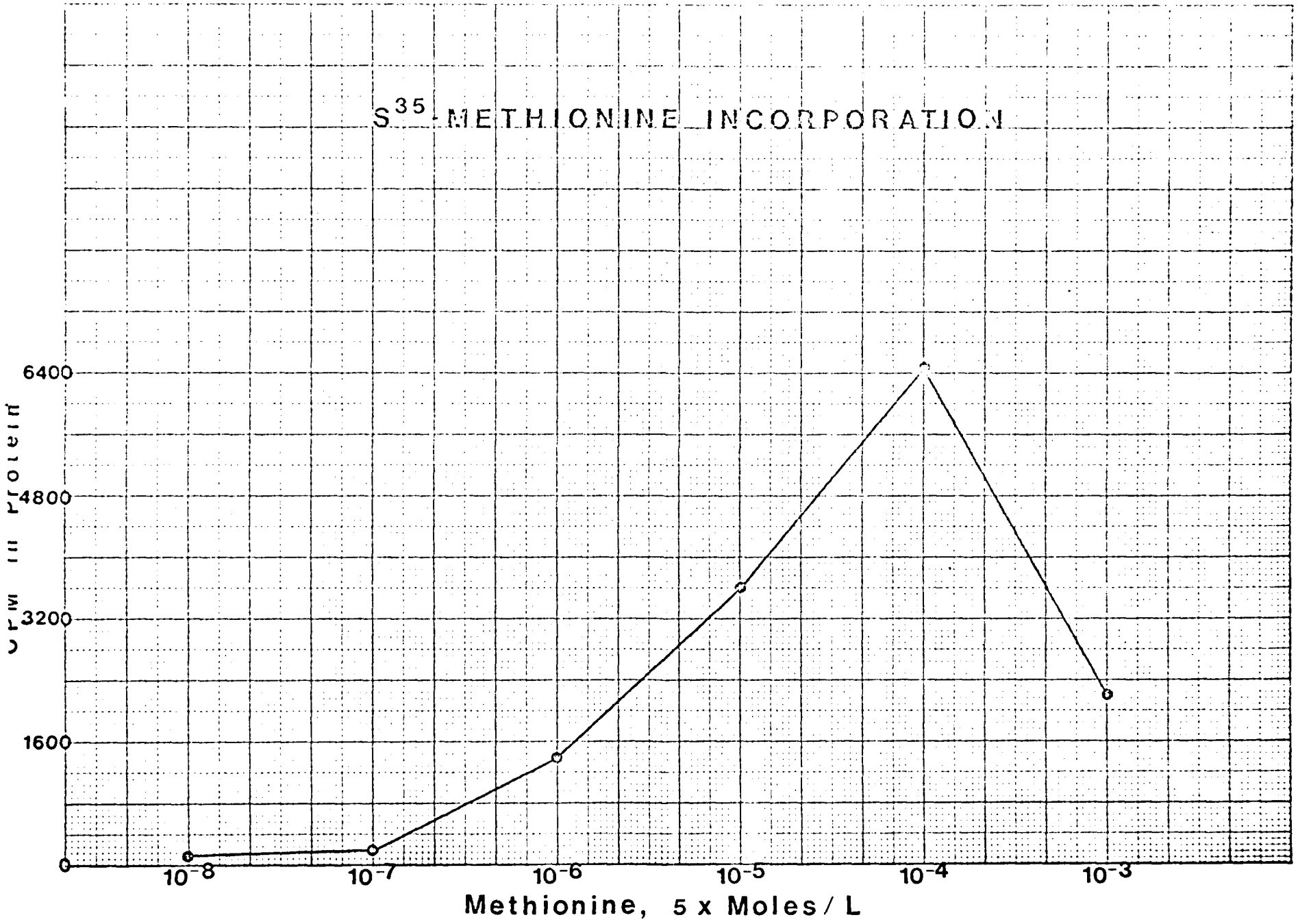
3. Demonstration that ^{35}S is not transferred from cysteine to methione. If ^{35}S-cysteine were converted to ^{35}S-methionine the calculated error level in the synthesis of 0.3 protein would be higher

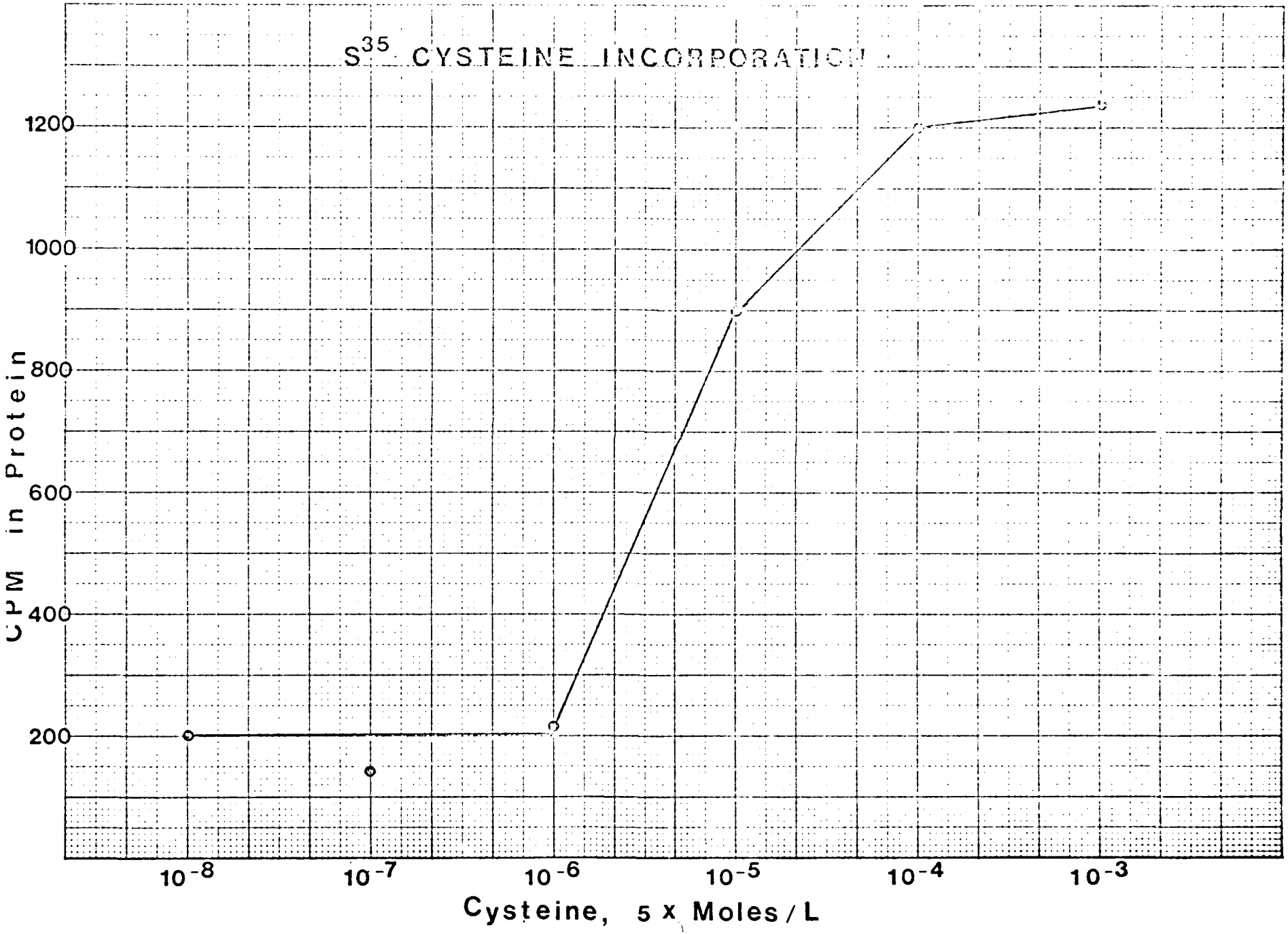
Cysteine Incorporation into 0.3 Protein
Increases with Increasing Gentamicin Concentration

FIGURE 5

Lanes	Gentamicin (ug/ml)
1a,1b	0
2a,2b	10
3a,3b	20
4a,4b	40

Lanes 1a, 2a, 3a + 4a are extracts of T7 infected E. coli B, labelled with ^{35}S-cysteine, and allowed to react with normal serum and then protein A Sepharose. Lanes 1b, 2b, 3b + 4b are extracts of T7 infected E. coli B, labelled with ^{35}S-cysteine and allowed to react with antiserum and then protein A Sepharose.


S35-METHIONINE INCORPORATION
CPM in Protein
6400
4800
3200
1600
0
10-8
10-7
10-6
10-5
10-4
10-3
Methionine, 5 x Moles / L


S35 CYSTEINE INCORPORATION
CPM in Protein
1200
1000
800
600
400
200
10-8
10-7
10-6
10-5
10-4
10-3
Cysteine, 5 x Moles / L

than the true error level since methionine is present in 0.3 protein. It is, therefore, necessary to show that methionine in the 0.3 protein contains no ^{35}S when ^{35}S-cysteine is supplied and that all the radioactivity is present in cysteine. Thin layer chromatography (TLC) was chosen as the best way to determine this. Several solvent systems and stationary phase solid supports were examined in preliminary experiments to determine the best way for separation of methioine and cysteine. We found that two-dimensional TLC provided no advantage over one-dimensional TLC and that N-propanol: ammonium hydroxide was the solvent system best suited to our needs. Silica gel, cellulose, or paper can be used as the solid support. The specific activity of ^{35}S-cysteine-labeled 0.3 protein is naturally very low and therefore long exposure times of the TLC chromatograms to x-ray flm are needed for satisfactory autoradiography.

We expect to obtain a precise determination of the normal error level for translation in vivo during the synthesis of 0.3 protein within the next few months.

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1974-1979 - Research Group Director, Max Planck Institüt für Molekulare Genetik, 1 Berlin 33, Germany. Responsible for control of recombinant DNA research.
1979 - Instructor in EMBO advanced techniques course (Plasmids), Erlangen, W. Germany.
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1. Reeve, J.N. 1979. Use of minicells for bacteriophage-directed polypeptide synthesis. Methods in Enzymol. 68:493-511.

2. Reeve, J.N. 1979. Isolation of minicells and characterization of plasmid and phage encoded polypeptides by SDS-polyacrylamide gel electrophoresis. EMBO Advanced Techniques Course on Plasmids. Erlangen, W. Germany. pp. 130-151.

3. Reeve, J.N., N. Trun and P. Hamilton. 1981. Beginning genetics of methanogens. In "Genetic Engineering of Micro-organisms for Chemicals." Ed. A. Hollaender. Asoc. Univ. Press., Wash., D.C. 20036. In Press.

CURRICULUM VITAE

July, 1982

NAME: Jacqueline Bowers Rice

PII Redacted [redacted]

EDUCATION:

B.A. University of Wisconsin-Milwaukee, 1960.

Registered Medical Technologist, ASCP, 1961
Milwaukee Lutheran Hospital Internship, 1960-1961.

Graduate Program, Department of Medical Microbiology, 1967-1968, Marquette University, Milwaukee, Wisconsin.

Graduate Program in Molecular Biology, Department of Biology, Marquette University, Milwaukee, Wisconsin, 1968-1970.

Graduate Program, Department of Medical Microbiology, Ohio State University, Columbus, Ohio, 1972-1977.

Doctor of Philosophy: Research was directed by Dr. Raymond Lang whose work has involved several transplantation models.

Dissertation Title: Extraction of xenotransplantation antigens and their use in xenograft prolongation and studies of xenograft rejection.

TEACHING EXPERIENCE:

Medical Student Independent Study Program, Ohio State University, 1975. (Conducted laboratory exercises in medical microbiology and infectious diseases.)

Laboratory Instructor for the medical student Medical Microbiology course (MM 625), Ohio State University, 1973.

Teaching Assistant (Genetics), Department of Biology, Marquette University, 1969.

APPOINTMENTS:

Research Associate II, Ohio State University, Department of Microbiology, College of Biological Sciences, 1981-present.

Research Associate I, Ohio State University, Department of Veterinary Pathobiology, College of Veterinary Medicine, 1977-1981.

Graduate Research Associate, Ohio State University, Department of Medical Microbiology, 1973-1975.

Medical Technologist, Mount Carmel Hospital, Columbus, Ohio, 1971.

Medical Technologist, Deaconess Hospital, Milwaukee, Wisconsin, 1970.

NIH Traineeship in Developmental Biology, 1968-1970.

NDEA Title IV Fellowship (NIH), 1967-1968.

Medical Technologist, Milwaukee County Hospital, Milwaukee, Wisconsin, 1964-1967.

Medical Technologist, San Diego County Hospital, San Diego, California, 1961-1963.

HONORS AND AWARDS:

Science Honorary Fraternity (Delta Chi Sigma), University of Wisconsin-Milwaukee, 1958-1960.

Graduated With Honors, University of Wisconsin-Milwaukee, 1960.

NDEA Title IV Fellowship (NIH), Marquette University, 1967-1968.

NIH Traineeship in Developmental Biology, 1968-1970.

GRANTS AWARDED:

USPHS, NIH, 1-RO1-CA30338-01, FeLV leukemogenesis and pre-neoplastic lesions, co-investigator, 3 yrs., $180,895 total, 11/81-10/84. (I wrote the entire grant proposal. Richard G. Olsen is listed as P.I. because of University regulations.)

The Ohio State Canine Research Funds, "Immunoprevention of Parvovirus-Induced Diarrhea of the Dog", 2 yrs., $20,000/yr., 1981-1982. (I wrote the entire grant proposal. Richard G. Olsen and G. Stephen Krakowka are listed as P.I.'s because of University regulations.)

PUBLICATIONS:

Rice, J.B.: Extraction of xenotransplantation antigens and their use in xenograft prolongation and studies of xenograft rejection. Ph.D. dissertation, 1977.

Rice, J.B. and Lang, R.W.: Solubilization and partial characterization of rat lymphocyte antigen. American Society for Microbiology, abstract, 1976.

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Krakowka, S., Olsen, R.G., Axthelm, M.K., Rice, J.B., and Winters, K.A.: Canine parvovirus infection potentiates canine distemper encephalitis attributable to modified live virus vaccine. J. Am. Vet. Med. Assoc., 180:137-139, 1982.

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